Volume 161, number 1 FEBS 0760 September 1983

Decrease in apparent K_m for oxygen after stimulation of respiration of rat polymorphonuclear leukocytes

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Received 4 July 1983

The respiratory burst of polymorphonuclear leukocytes, induced by the addition of chemotactic peptide (*N*-formyl-methionyl–leucyl–phenylalanine) and cytochalasin B was found to consist of two phases. The first phase of very rapid oxygen uptake lasted 1–3 min. and was followed by a second more prolonged phase of lower magnitude. The apparent K_m for oxygen of unstimulated cells was 9.6 ± 0.67 μM, while that of the second phase of stimulation was 3.7 ± 1.6 μM oxygen. The possibility that lowered oxygen concentrations may regulate polymorphonuclear leukocyte activity in some pathological conditions is discussed.

Polymorphonuclear leukocyte, decrease in app. K_m for oxygen after stimulation Respiration burst, in polymorphonuclear leukocytes Oxygen affinity, of stimulated polymorphonuclear leukocytes respiration Chemotactic peptide, effect on polymorphonuclear leukocytes respiration Cytochalasin B, enhancement of effect of chemotactic peptide on polymorphonuclear leukocyte respiration

1. INTRODUCTION

The respiratory burst of polymorphonuclear leukocytes (polymorphs) involves an increased rate of oxygen uptake and an increase in glucose oxidation via the hexose monophosphate shunt [1]. This dramatic change in metabolic activity is accompanied by the production of oxygen radicals and may be induced by a number of stimuli. These include: opsonized bacteria or latex beads [2]; opsonized zymosan [3]; chemotactic factors [4]; antibody plus complement [5,6]. Luminol-dependent chemiluminescence provides a highly sensitive and continuous method for the monitoring of the rate of production of oxygen radicals by cells [7]. These radicals are produced by the reaction of oxygen with an NAD(P)H oxidase system [8,9], but the precise relationship and mechanisms involved are not fully understood [10]. Indeed, the terminal components of this NAD(P)H oxidase system have yet to be unequivocally identified, although a unique *b*-type cytochrome is thought to be involved [11] which is capable of reducing molecular oxygen to O_2^- [12].

Despite the appreciation that gradients of oxygen exist in tissues [13], there have been surprisingly few detailed studies on the activities of oxygen-requiring enzymes other than cytochrome $a + a_3$. In particular, the relationships between the production of the various species of oxygen radicals, the respiratory burst and oxygen tension during phagocytosis and chemotaxis in polymorphs are unknown. The aim of the work presented here was to investigate the relationship between oxygen concentration and polymorph respiration in the presence and absence of stimuli. These results show a decreased apparent K_m for oxygen after stimulation. Furthermore under steady-state conditions the respiratory burst appeared to have two components, one of which was short-lived (1–3 min) whilst the other was continued for at least 30 min.

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2. MATERIALS AND METHODS

Rat polymorphonuclear leukocytes were prepared from intraperitoneal fluid exactly as in [6] following intraperitoneal injection of sodium caseinate. After purification they were suspended in a buffer containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.1% BSA and 25 mM HEPES (pH 7.4) and used within 6 h of preparation.

Oxygen uptake was measured polarographically at 37°C on suspensions of cells either in a closed system fitted with a Clark type oxygen electrode or in an 'open' system [14] based on that described in [15]. In the latter system the working volume was 8.0 ml and the oxygen tension in the gas phase was controlled by a digital gas mixer allowing mixtures of air and argon in 5% steps: oxygen tension in the liquid phase was monitored with a membrane-covered electrode (type D602; Radiometer, Copenhagen). With a constant partial pressure of oxygen in the gas phase and the attainment of a steady-state level in the liquid phase, the respiration rate (V_r) was calculated from the equation $V_r = K(T_G - T_L)$ where K is the oxygen transfer constant (min^{-1}), T_G is the oxygen tension in the gas phase and T_L is the oxygen tension in the liquid phase [16].

3. RESULTS

3.1. *Effects of chemotactic peptide (N-formyl-methionyl-leucyl-phenylalanine) and cytochalasin B on respiration of rat polymorphonuclear leukocytes*

The resting respiration rate of rat polymorphonuclear leukocytes, measured in the closed oxygen electrode, was found to be about 3.5 nmol oxygen/min/10^7 cells (fig.1a). Upon the addition of 1 μM (final conc.) chemotactic peptide and 1.5 μg cytochalasin B (added together), the respiratory rate initially increased to 19.6 nmol/min/10^7 cells. This elevated respiration rate lasted 1–3 min and then returned to just above the resting level. Addition of chemotactic peptide alone (1 μM) again produced a rapid, although small, increase (fig.1b) in respiration rate which was about 50% above the resting rate; addition of 1.5 μg cytochalasin B added later did not significantly increase this. Addition of cytochalasin B alone did not increase the respiration rate (fig.1c) but a further addition of chemotactic peptide resulted in an initial 4.9-fold increase (maintained for about 2 min) followed by a lower, more sustained increase.

The effects of chemotactic peptide and cytochalasin B on respiration of rat polymorphonuclear leukocytes measured in the 'open' electrode are shown in fig.2. With 40% air:60% argon as the mixture in the gas phase, a steady-state level of respiration (3.7 nmol oxygen/min/10^7 cells) was indicated by a constant level of oxygen in the liquid phase. When 1 μM chemotactic peptide (final conc.) and 5 μg cytochalasin B were added, the level of oxygen in the liquid phase decreased; since the oxygen concentration in the gas phase remained constant this represented an increased respiration rate. This elevated respiration rate was not

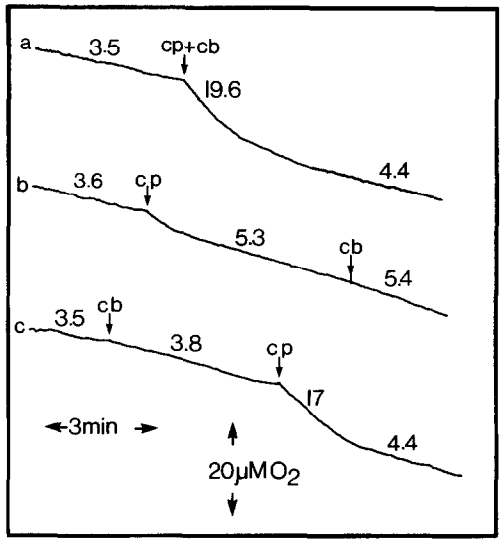

Fig.1. Effects of chemotactic peptide (*N*-formyl-methionyl-leucyl-phenylalanine) and cytochalasin B on the respiration of rat polymorphonuclear leukocytes. A suspension of freshly prepared rat polymorphonuclear leukocytes was diluted to 5 × 10^6 cells/ml in buffer (pH 7.4) and placed in the oxygen electrode: the total volume was 1.5 ml. After allowing for the endogenous respiration to be measured, additions were made as indicated; cp = the addition of 1 μM (final conc.) chemotactic peptide (*N*-formyl-methionyl-leucyl-phenylalanine); cb = the addition of 1.5 μg of cytochalasin B. Numbers refer to respiration rates measured from the traces as indicated as nmol O_2/min/10^7 cells. Traces a,b and c were obtained from 3 separate suspensions of cells.

maintained, however, but by about 14 min had stabilised at 5.1 nmol/min/10^7 cells. A further addition of chemotactic peptide and cytochalasin B did not result in further stimulation. This stimulated respiration rate (about 40% above resting rate) was maintained for >30 min (not shown).

3.2. *Apparent* K_m *for oxygen of respiration of stimulated and unstimulated rat polymorphonuclear leukocytes*

A suspension of rat polymorphonuclear leukocytes (1.8 $\times 10^7$/ml; total vol. 8.0 ml) was placed in the 'open' oxygen electrode. Initially the gas phase consisted of 100% air and a steady-state level of oxygen in the liquid phase was attained (fig.3a). The oxygen concentration in the gas phase was then varied using a digital gas mixer to allow calculation of respiration rates at various air–argon mixtures, under steady-state conditions. Similar measurements were made on cells stimulated by the addition of 1 μM chemotactic peptide (final conc.) and 5 μg cytochalasin B (total vol. 8.0 ml) as shown in fig.3b. From the calculations of respiration rates at various oxygen concentrations, Michaelis Menten kinetics were fitted from a computer program. The apparent K_m-value for oxygen of unstimulated cells was 9.6 ± 0.67 μM and for stimulated cells was 3.7 ± 1.6 μM (number of determinations = 3): V_{max} for respiration was increased by 51.7 ± 10% after stimulation.

4. DISCUSSION

Chemotactic factors have been shown to induce a number of responses in polymorphs, including a respiratory burst [4]. We have resolved this respiratory burst in two phases which are a transient initial phase followed by a more prolonged second phase of lower magnitude. We report for the first time that the apparent K_m for oxygen of this second phase is 3-times lower than that measured in resting cells: the initial phase was too transient to allow accurate measurement of apparent K_m. The addition of cytochalasin B (an inhibitor of microtubule formation, [17]), together with or before the addition of chemotactic peptide, increased both the magnitude and duration of this initial phase. The mechanism of this enhancement by cytochalasin B is not known, although its combined effect with chemotactic peptide also increases granule enzyme secretion [4] and potentiates luminol-dependent chemiluminescence [18,19].

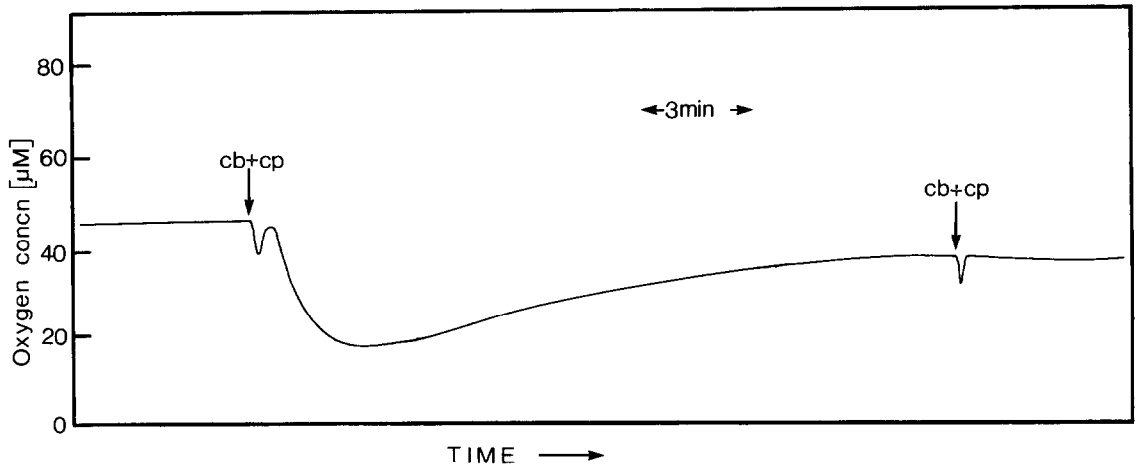

Fig.2. Effects of cytochalasin B and chemotactic peptide (*N*-formyl-methionyl-leucyl-phenylalanine) on the respiration of rat polymorphonuclear leukocytes measured in an 'open' oxygen electrode. A suspension of rat polymorphonuclear leukocytes in buffer (5 × 10^7 cells/ml) was placed in the chamber of the 'open' oxygen electrode: the total volume was 8.0 ml. With 40% air as the gas phase (controlled by a digital gas mixer) a steady-state level of respiration was attained and then, as indicated (↓), 5 μg cytochalasin B and 1 μM (final conc.) chemotactic peptide were added. The trace shows measured oxygen concentration in the liquid phase, which decreases as respiration rate increases.

This latter response results from the production of oxygen radicals and it is thus tempting to suggest that this enhancement of oxygen uptake is related to increased oxygen radical production.

The second phase is more clearly seen with the 'open' oxygen electrode (fig.2) where steady-state levels of respiration are measured at fixed oxygen tensions. This apparatus is thus particularly well suited to the measurement of app. K_m-values. App. K_m-values for oxygen of whole cell respiration of a number of different cell types have been measured by a number of methods. Typically in cells where mitochondrial cytochrome $(a + a_3)$ is the sole terminal oxidase, apparent K_m-values are 1 μM oxygen or less [20]. For values below this, polarographic measurements become unreliable and more sensitive methods using bacterial luminescence are required [21–23]. Mitochondrial respiration did not contribute significantly to oxygen uptake in resting or stimulated rat polymorphs, since the addition of 1 mM sodium azide inhibited both stimulated and resting respiration by less than 10%. While the apparent K_m for cellular respiration of isolated hepatocytes is about 2 μM O_2, other oxygen-requiring reactions in these cells such as cytochrome P-450 functions show an even lower affinity [24]. Although the oxygen concentration in normal blood varies over the range 120–50 μM [25], it has been proposed that considerable oxygen gradients exist in tissues and at

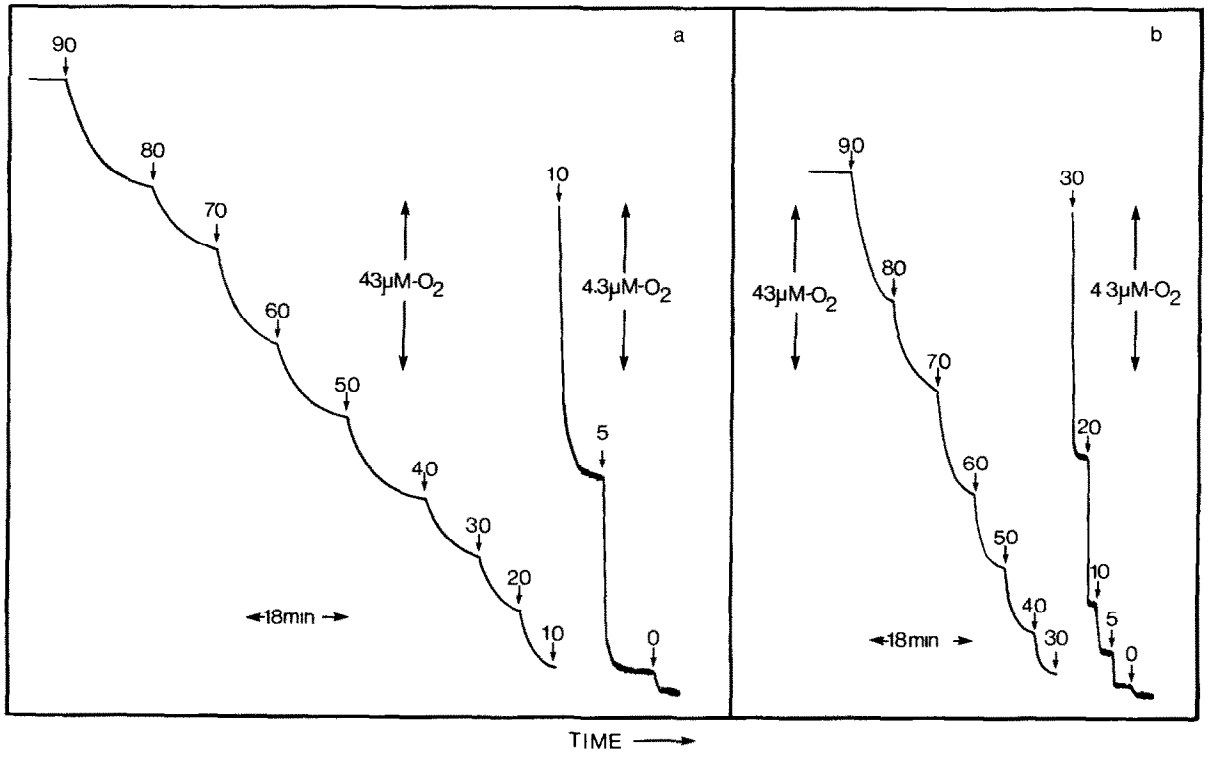

Fig.3. Measurements of oxygen uptake rates of rat polymorphonuclear leukocytes as a function of oxygen concentration in the 'open' oxygen electrode. A suspension of rat polymorphonuclear leukocytes (1.8×10^7 cells/ml) in buffer (pH 7.4) was placed in the 'open' oxygen. A steady-state level of respiration was achieved with the gas phase consisting of 100% air. The oxygen concentration in the gas phase was then controlled using a digital gas mixer to give mixtures of air and argon as indicated: figures at ↓ represent the % of air in the mixture; ○ represents 100% argon in the gas phase. (a) Unstimulated cells. When the gas phase contained 10% air, oxygen levels were recorded at a 10-fold higher sensitivity. (b) Cells stimulated by the addition of 1 μM (final conc.) chemotactic peptide (*N*-formyl-methionyl-leucyl-phenylalanine) and 5 μg cytochalasin B 5 min before measurements were made. When the gas phase contained 30% air, oxygen levels were recorded at a 10-fold higher sensitivity.

sites of inflammation oxygen concentration can be less than 12 μM [26]. Our results emphasize the need for careful characterization of the app. K_m-values of oxygen requiring enzymes, particularly those which are relatively high. The possibility now exists that these enzymes may be regulated under physiological and pathological conditions by oxygen availability. Polymorph infiltration into tissues occurs during acute inflammation and in several pathological conditions; e.g., rheumatoid arthritis and after myocardial infarction. We are now examining the relationship between oxygen concentration and radical production and its significance in the pathogenesis of these conditions.

ACKNOWLEDGEMENTS

We thank the Science and Engineering Research Council and the Arthritis and Rheumatism Council for financial support.

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